

High-Purity Bacterial RNA Isolated with the Agilent Total RNA Isolation Mini Kit

Application

Gene Expression

Author

Christopher Rizzo
Claudia A. Robbins
Rhonda R. Taylor
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington DE 19808-1610
USA

Abstract

A protocol for the isolation of high-purity RNA from gram-positive and gram-negative bacteria was developed for use with the Agilent Total RNA Isolation Mini Kit. High-purity RNA is characterized by the absence of cellular contaminants, such as genomic DNA. Genomic contamination is especially problematic in reverse transcription-polymerase chain reaction (RT-PCR) experiments from bacterial RNA because of the absence of introns in the bacterial genome. Other contaminants carried downstream into enzymatic reactions, such as target labeling or PCR, could interfere with reaction efficiency and complicate interpretation of experimental results. RNA isolation from gram-positive bacteria is complicated by a cell wall that is difficult to disrupt. The yield of bacterial RNA isolations depends on the appropriate use of lysozyme treatment. Under appropriate conditions, we have observed a five-fold increase in yield and orders of magnitude less genomic DNA contamination, as measured by UV absorbance and quantitative PCR respectively.

Introduction

The Agilent Total RNA Isolation Mini Kit (p/n 5185-6000) is a phenol-free spin-column method for the isolation of total RNA (Figure 1). This method employs a unique prefiltration column, resulting in the removal of cellular contaminants including genomic DNA (gDNA). Use of this kit results in RNA with low gDNA contamination levels without DNase treatment.

The original mammalian cell and tissue RNA isolation protocols were modified for improved results when isolating RNA from bacteria. With this optimized protocol, RNA isolated from gram-positive and gram-negative bacteria is of high yield and purity. Lysozyme conditions were optimized to maximize yield from gram-positive bacteria.

Following enzymatic treatment, the lysate is centrifuged through the prefiltration column. Partially purified RNA is then mixed with ethanol, incubated, applied to the membrane-based isolation column, and centrifuged. After washing, the RNA is eluted from the isolation column with nuclease-free water in a volume as low as 10 μ L.

Use of this kit often eliminates the need for DNase digestions. This is particularly significant when performing RT-PCR experiments from bacterial RNA. The absence of introns in the bacterial genome eliminates the ability to distinguish between the mRNA and gDNA signals in PCR. Bacterial RNAs are often treated with DNase I to minimize gDNA contamination and many times



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multiple DNase treatments are needed. Minimizing the gDNA contamination in bacterial RNA isolations can eliminate the added expense and hands-on time associated with DNase treatments.

This application note describes the isolation of RNA from gram-positive and gram-negative bacteria and the analysis for RNA quality via the Agilent 2100 Bioanalyzer, a microfluidics-based platform for the analysis of RNA, as well as DNA, protein and cells. Yield was determined via A_{260nm} and gDNA contamination was determined via a quantitative PCR assay.

Materials and Methods

Bacterial strains were obtained from ATCC (Manassas, VA). Liquid cultures were incubated at 30 °C overnight (*B. subtilis*) or 37 °C for 3 hours (*E. coli*), shaking at 200 rpm. Serial dilutions were plated, cell pellets collected and frozen at -80 °C. All RNA isolations were from 5×10^8 cells.

RNA was isolated with the Agilent Total RNA Isolation Mini Kit following the detailed protocol in Appendix A, or by a silica-based method (QIAGEN RNeasy and RNase-free DNase Set, QIAGEN, Valencia, CA). RNA quality was determined via the Prokaryotic Total RNA Nano assay from the RNA 6000 Nano Labchip® Kit using the Agilent Technologies 2100 Bioanalyzer. RNA was quantitated by A_{260nm} on the ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Genomic DNA contamination was detected using quantitative PCR on the ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City, CA) using primer/probe sets specific to GAPDH (Biosearch Technologies, Novato, CA) and purified gDNA (ATCC) as a standard.

Results

The results from the Prokaryotic Total RNA 6000 Nano assay are shown in Figure 2 and Figure 3. Although the *B. subtilis* RNA isolated with the silica-based kit is intact as demonstrated by appropriate 16S and 23S peaks, there is visible gDNA contamination as well as extraneous signal. Clarification of the homogenate prior to the addition of ethanol does not improve the quality of the RNA. On-column DNase treatment does eliminate the signal from the gDNA. The *B. subtilis* RNA isolated with the Agilent protocol exhibits a relatively flat baseline with 16S and 23S RNA peaks. *E. coli* RNA isolated with both kits results in electropherograms typical of intact prokaryotic RNA.

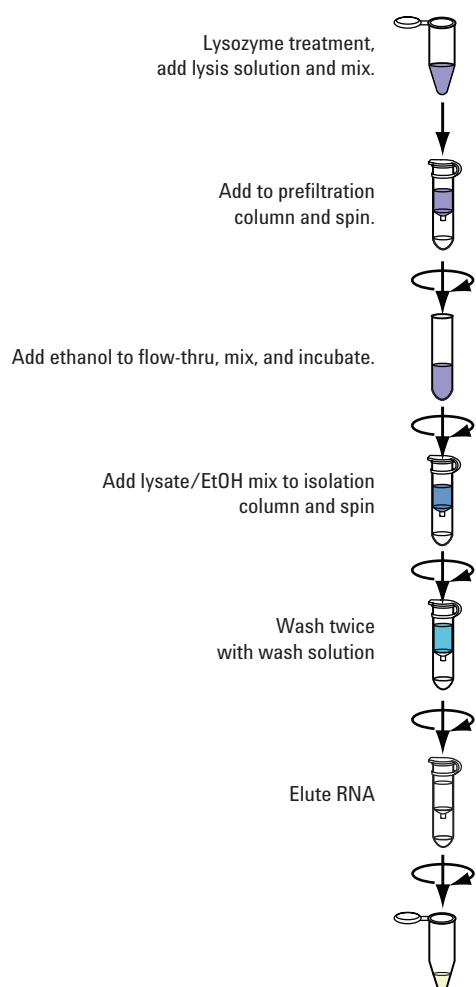


Figure 1. Agilent Total RNA Isolation Method for isolating bacterial RNA.

Quality of *B. subtilis* RNA Isolations

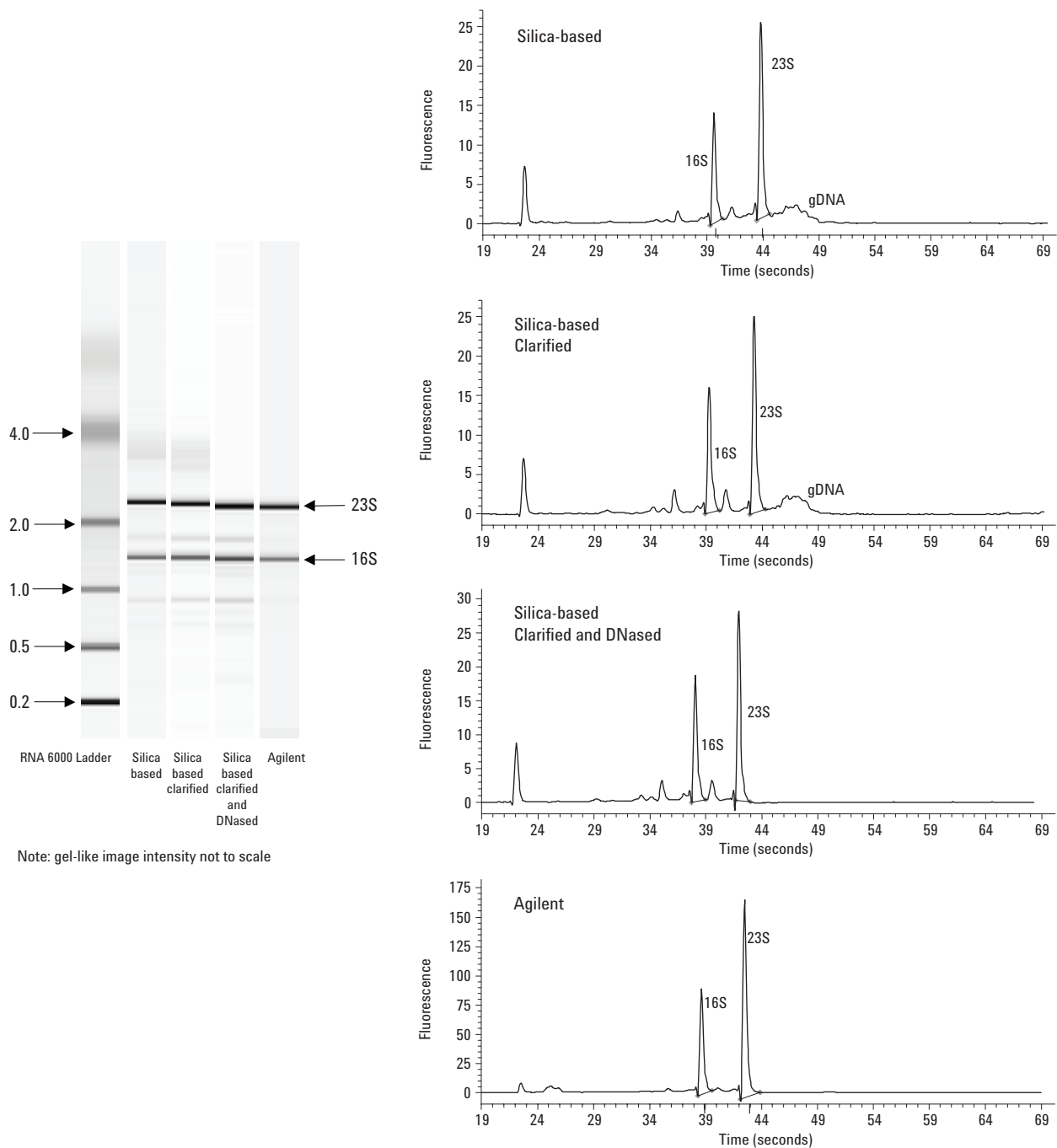


Figure 2. Gel-like images and electropherograms of total RNAs isolated from *B. subtilis* RNA were isolated using a silica-based kit or the Agilent protocol.

Quality of *E. coli* RNA Isolations

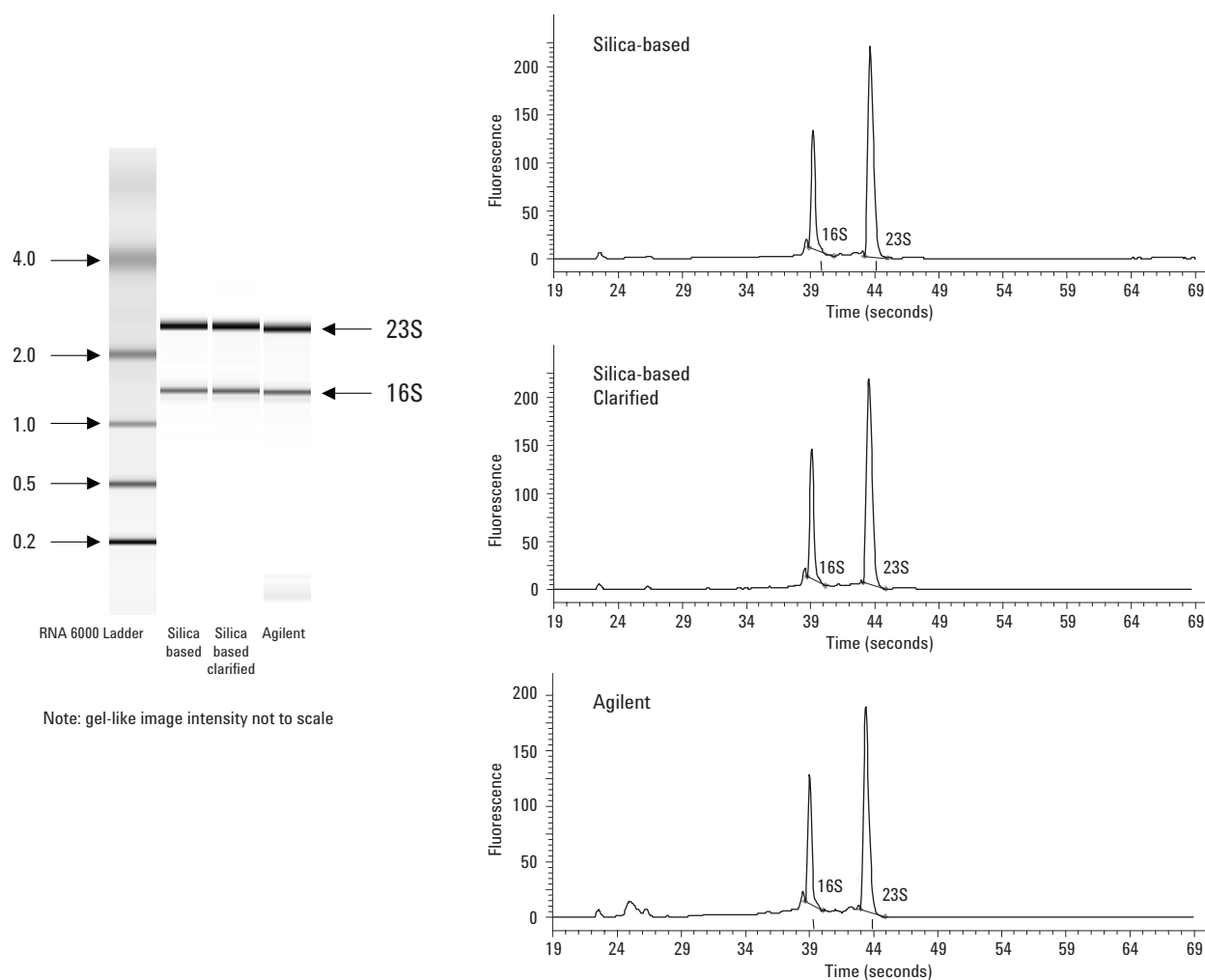


Figure 3. Gel-like images and electropherograms of total RNAs isolated from *E. coli* RNA were isolated using a silica-based kit or the Agilent protocol.

RNA was quantitated by $A_{260\text{ nm}}$. All $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratios were 1.9 or greater. The yields are shown in Figure 4. When isolating RNA from gram-positive bacteria, clarification of the lysate in the silica-based isolations increases yield to a small extent. For these RNA isolations, increasing the time of lysozyme treatment to 40 minutes increases the yield an average of five-fold compared to the silica-based protocol. Using either kit, RNA isolations from gram-negative bacteria result in sufficient and equivalent yield.

Genomic DNA contamination was detected via a quantitative PCR assay (Figure 5). *B. subtilis* RNA isolated with the silica-based kit results in high gDNA contamination, approximately 1000-fold higher than RNA isolated with the Agilent Kit. On-column DNase treatment with the silica-based kit lowers this contamination to the levels of RNA isolated with the Agilent kit, which is not DNase treated. *E. coli* RNA isolated with the silica-based kit also results in gDNA contamination levels 1000-fold higher than RNA isolated with the Agilent kit.

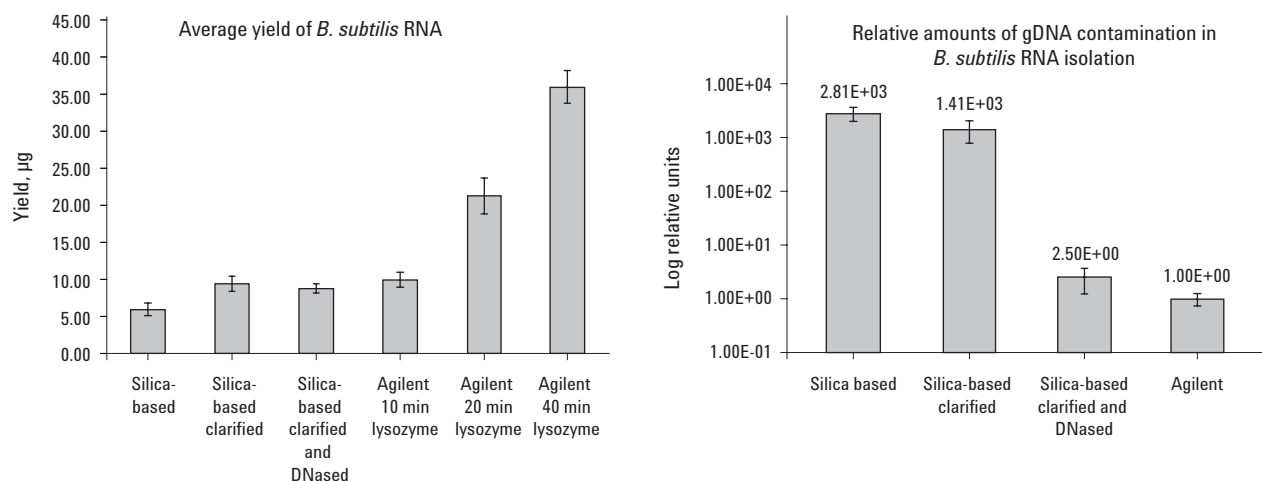


Figure 4. Yields of total RNAs and relative amounts of gDNA contamination in total RNAs isolated from *B. subtilis*. RNA was isolated using a silica-based kit or the Agilent protocol.

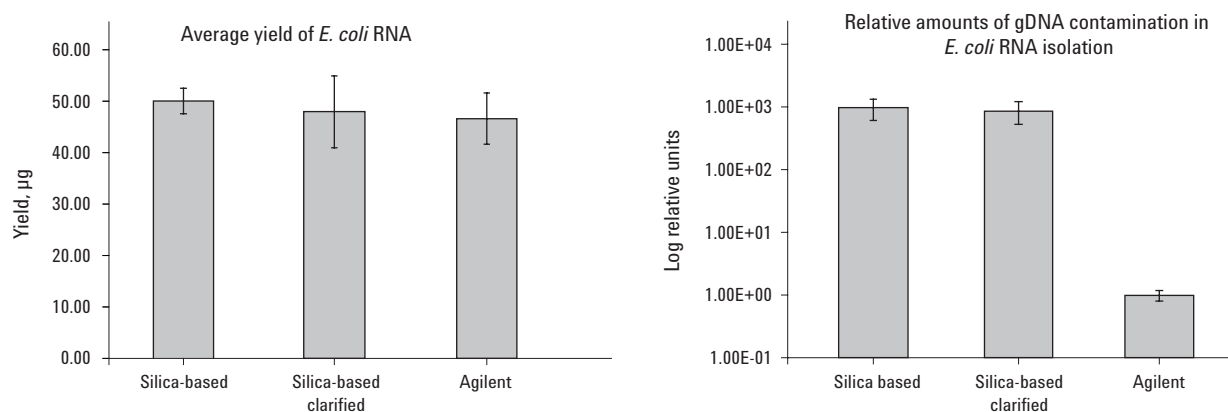


Figure 5. Yields of total RNAs and relative amounts of gDNA contamination in total RNAs isolated from *E. coli*. RNA was isolated using a silica-based kit or the Agilent protocol.

Conclusions:

The Agilent Total RNA Isolation Mini Kit provides a fast and efficient, phenol-free, spin-column based method for the preparation of concentrated, high-purity, intact total-cellular RNA.

- RNA isolated from gram-positive and gram-negative bacteria is of high-yield.
- Levels of gDNA contamination are extremely low, even when compared to RNA treated with DNase from a silica-based kit.
- RNA was of higher quality as analyzed by the Agilent 2100 Bioanalyzer.
- DNase treatment of RNA isolations can be eliminated when using the Agilent kit, simplifying workflow and reducing the risk of sample contamination or loss.

A

Bacterial RNA Isolation

This protocol is for use with the Agilent Total RNA Isolation Mini Kit (p/n 5188-6000).

Additional Materials Required

In order to perform Bacterial RNA isolations, you will also need:

- Microcentrifuge
- 100 % Ethanol
- 14.3 M β -Mercaptoethanol (β -ME)
- Lysozyme
- TE, pH 8.0

Reagent Storage

The Agilent Total RNA Isolation Mini Kit should be stored at room temperature prior to opening. Lysis solution should be stored at 4 °C following the addition of (β -ME).

Before You Begin

Bacterial cultures are to be grown overnight at 30 °C or for a few hours (typically 3 hours) at 37 °C. Growth conditions are dependent on the particular strain used. Avoid isolating RNA from saturated cultures. OD_{600nm} readings can approximate culture density (a reading of 1 at 600 nm is approximately 10⁹ bacteria). Serial dilutions of culture should be plated to confirm culture density at a particular absorbance reading.

Centrifuge cultures at 4 °C between 3,000–6,000 × g. The growth medium must be thoroughly removed from the cell pellet by draining or aspirating. Additionally, an absorbent material can be used to remove final traces of medium from the centrifuge tube or bottle. The cell pellet can be frozen at –80 °C.

Lysozyme from lyophilized powder is made in TE pH 8.0 just prior to use. Lysozyme should be 400 μ g/mL for isolations from gram-negative strains and 3 mg/mL for gram-positive strains. Using frozen aliquots of lysozyme may result in reduced yields.

To prepare Lysis Solution, add 500 μ L of β -ME to the bottle labeled lysis solution and mix well. To prepare smaller amounts, add 10 μ L of β -ME per 1 mL lysis solution.

To prepare the Wash Solution, add 48 mL of ACS grade 100% Ethanol to the bottle provided labeled Wash Solution and mix well.

The capacity of the isolation column is approximately 10⁹ cells or 100 μ g total RNA.

Carry out all steps at room temperature. Use RNase-free technique.

Agilent Total RNA Isolation Mini Kit Information

For additional information regarding the Agilent Total RNA Isolation Mini Kit, please refer to the Instruction Manual (p/n 5188-2710).

Isolation Protocol

1. Resuspend pellet in 100 μ L of Lysozyme prepared in TE pH 8.0. Gram-negative cells should be digested for 5–20 minutes and gram-positive cells should be digested for 20–40 minutes.
2. Add 200 μ L of prepared Lysis Solution to digested cells and mix well by pipetting. Add this mixture to a prefiltration column and centrifuge for 3 minutes at 16,000 × g.
3. Add 300 μ L of 100% ethanol to the flow-through from the prefiltration column. Mix well by pipetting and incubate for 5 minutes.

4. Add the lysate/ethanol mixture to an isolation column, centrifuge 30 s at $16,000 \times g$. Remove and dispose of flow-through.
5. Add 500 μL prepared Wash Solution to isolation column, centrifuge 30 s at $16,000 \times g$. Remove and dispose of flow-through. Repeat this step for a total of two washes with Wash Solution.
6. Centrifuge isolation column for 2 minutes at $16,000 \times g$ to remove final traces of Wash Solution.
7. To elute RNA: add 10–50 μL nuclease-free H_2O to center of membrane in isolation column. Incubate 1 minute and centrifuge 1 minute at $16,000 \times g$.

NOTE: If more concentrated RNA samples are desired for downstream application, the elution volume may be as low as 10 μL . However, if the final RNA concentration exceeds 3 $\mu\text{g}/\mu\text{L}$, quantitative recovery of the RNA may be compromised. The expected yield must be taken into consideration when choosing an elution volume. If the expected yield is not known, use 25 μL nuclease-free water for elution and determine the concentration by $A_{260\text{nm}}$. If the concentration is greater than 3 $\mu\text{g}/\mu\text{L}$, residual RNA may be remaining on the membrane. Apply an additional 25 μL to isolation column, centrifuge 1 min $16,000 \times g$ and determine concentration of the second elution. If significant RNA is present, then pool with original elution.

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